



## REVIEW ARTICLE



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# Overview of in vivo and ex vivo endpoints in murine food allergy models: Suitable for evaluation of the sensitizing capacity of novel proteins?

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## Abstract

Significant efforts are necessary to introduce new dietary protein sources to feed a growing world population while maintaining food supply chain sustainability. Such a sustainable protein transition includes the use of highly modified proteins from side streams or the introduction of new protein sources that may lead to increased clinically relevant allergic sensitization. With food allergy being a major health problem of increasing concern, understanding the potential allergenicity of new or modified proteins is crucial to ensure public health protection. The best predictive risk assessment methods currently relied on are in vivo models, making the choice of

**Abbreviations:** Ag, allergen; ALA, alpha-lactalbumin; BAT, basophil activation test; BLG, beta-lactoglobulin; BN, Brown Norway; DC, dendritic cell; e.c., epicutaneously; ELISA, enzyme-linked immunosorbent assay; GM-CSF, granulocyte-macrophage colony-stimulating factor; i.c., intracutaneous; i.d., intradermally; i.g., intragastric; i.n., intranasal; i.p., intraperitoneally; Ig, immunoglobulin; ILC, innate lymphoid cell; i.v., intravenous; KSCN, potassium thiocyanate; NA, not analysed; NKT, natural killer T cell; OVA, ovalbumin; PCR, polymerase chain reaction; RBL, rat basophil leukemia; s.c., subcutaneously; SPT, skin prick test; Treg, regulatory T cell; TSLP, thymic stromal lymphopoietin; WPC, whey protein concentrate; WPH, whey protein hydrolysate.

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endpoint parameters a key element in evaluating the sensitizing capacity of novel proteins. Here, we provide a comprehensive overview of the most frequently used *in vivo* and *ex vivo* endpoints in murine food allergy models, addressing their strengths and limitations for assessing sensitization risks. For optimal laboratory-to-laboratory reproducibility and reliable use of predictive tests for protein risk assessment, it is important that researchers maintain and apply the same relevant parameters and procedures. Thus, there is an urgent need for a consensus on key food allergy parameters to be applied in future food allergy research in synergy between both knowledge institutes and clinicians.

#### KEYWORDS

animal models, biomarkers, food allergy, prevention

## 1 | INTRODUCTION

A variety of *in vitro* and *in vivo* models have been developed that address the factors and mechanisms involved in the sensitization to food proteins.<sup>1–4</sup> Currently, approaches are being developed using protein chemistry and *in vitro* and *in silico* methods to characterize food proteins and derivatives that arise during product processing and reformulation, which may explain why certain food proteins induce sensitization of the immune system, while others are tolerated.<sup>5,6</sup> However, elucidating the mechanisms underlying allergen sensitization is a complex, multidimensional problem that often requires a wide range of additional *in vivo* and *ex vivo* experimentation,<sup>5</sup> as a wide range of molecules, tissues, and cells play a role in the mechanisms underlying food allergen sensitization.<sup>1</sup> For instance, epithelial release of thymic stromal lymphopoietin (TSLP), granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-25, and IL-33 upon local epithelial stress support type 2 helper T (Th2) cell pathology by attracting IL-4 secreting lymphoid cells, basophils, and invariant natural killer T (iNKT) cells.<sup>7</sup> IL-4 promotes surface expression of Th2-costimulatory molecule OX40 ligand

on dendritic cells (DCs)<sup>8</sup> and cytokine secretion by Th2 lymphoid cells (ILC2s), which further augments DC activity and suppresses allergen-specific regulatory T (Treg) cells.<sup>9,10</sup> This complexity, as depicted in Figure 1, illustrates the need for experimental food allergy models that integrate such complex cell-tissue communication to assess the sensitization potential of new protein sources. Murine food allergy models, even though they have their limitations, are currently the best predictive models available to evaluate the food-sensitizing capacity of new food proteins before introducing them into the human diet. Although researchers aim to reduce the use of experimental animals to address the 3R principle that guides animal experimentation to replace (alternative model), reduce (minimize number of animals), and refine (minimize animal pain and enhance animal welfare), there is a lack of replacement models such as *in silico* prediction models, *in vitro* primary cell assays, or tissue explants assays that are able to characterize and predict the human responses to food proteins. In the past, numerous experimental food allergy models have been developed to assess food allergenicity. However, interlaboratory differences in the models used with respect to sensitization and elicitation route, choice of

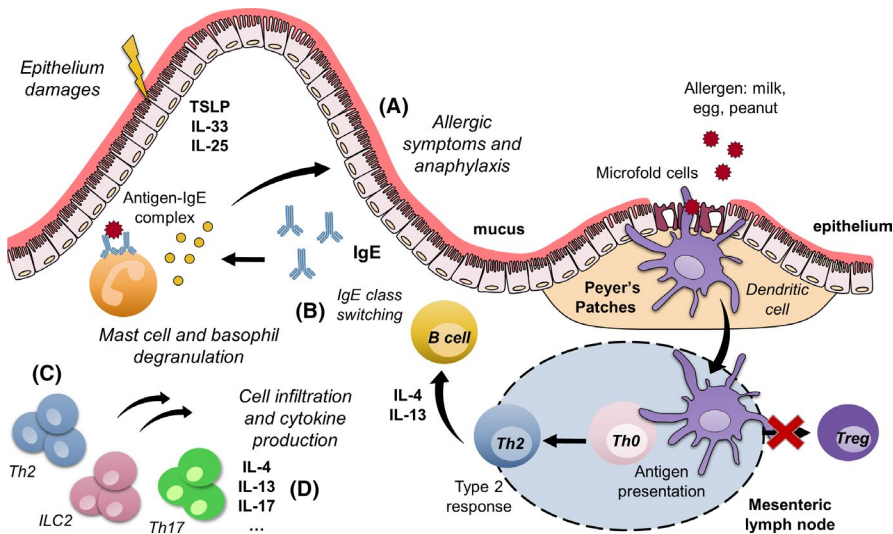
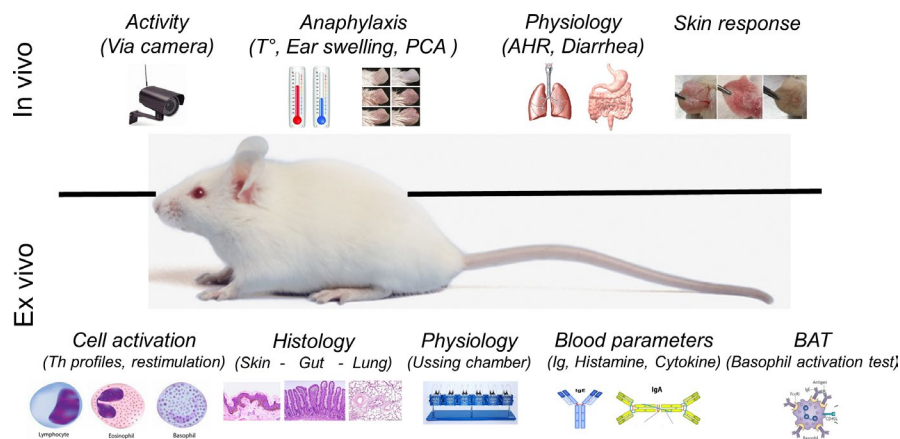


**FIGURE 1** Immune mechanisms of food allergy and its associated principal measured endpoints. A, Assessment of allergic symptoms (body temperature) after allergen challenge. B, Evaluation of immunoglobulin (IgE) in serum. C, Phenotyping of T-cell population. D, Cytokine production in response to allergen restimulation (*ex vivo* assay)

**FIGURE 2** In vivo and ex vivo methodological endpoints used in murine food allergy models



adjuvant, clinical signs, genetic background of the animals, housing conditions, and microbiome composition and metabolic activity in the different vivaria often make it difficult to draw generalized conclusions.<sup>5</sup> It is important to note that almost all models (except genetic models) require adjuvants to trigger sensitization. Therefore, the choice of the adjuvants together with the exposure route are crucial points to consider. In addition, there are numerous in vivo, ex vivo, and in vitro parameters evaluated for the assessment of food allergy. Figure 2 illustrates the types of in vivo (inside a living organism) or ex vivo (outside an organism) methodology and endpoints used in experimental murine models of food allergy. However, there is a need to establish a list of reliable, validated, and effective endpoint parameters to guide researchers working with animal models of food allergy. In this review, we describe a selective list of the most commonly used experimental applied endpoints in food allergies with a focus on milk, egg, and peanut allergens and critically evaluate their applicability for evaluating sensitization potency. Each endpoint was selected and critically described with strengths and limitation based on consortium experience and occurrence in literature.

## 2 | MEASUREMENT OF BODY TEMPERATURE

In murine-type models of food allergy to milk, eggs, and peanuts, a drop in the core body temperature is often observed after repetitive allergen challenge. This change in body temperature is an indicator of anaphylaxis (Table 1). Temperature is measured before and 30 minutes to 1 hour after allergen challenge, but this parameter can also be monitored over time.<sup>21,22</sup> Animals sensitized to a given food matrix or protein may display a significant reduction in body temperature (0.5–10°C)<sup>3,4</sup> compared with that of naive animals. For an adequate level of sensitivity, 5–16 animals per group should have their temperatures measured using a rectally inserted thermal probe,<sup>29</sup> but it is also possible to measure changes over time for individual animals using an electronic ID transponder implanted subcutaneously.<sup>11,12</sup> To refine, improve, and objectify

the currently applied manual monitoring methods, an automatic imaging method has been developed.<sup>14</sup> It involves a noninvasive measurement of the whole-body surface temperature paired with assessment of activity (see also Data S1 about activity/behavior via camera). Anaphylaxis imaging has been used in three in vivo allergy mouse models for (a) milk allergy, (b) egg allergy, and (c) peanut allergy in proof-of-principle experiments and suggests that imaging technology represents a reliable noninvasive method for objective monitoring of small animals during anaphylaxis over time. This method can be useful for monitoring diseases associated with changes in both body temperature and physical behavior.

### 2.1 | Strengths

- The measurement of core body temperature is a cost-effective, reliable assessment of the allergic reaction.
- Therapeutic or preventative strategies for the reduction of allergic reactions can be easily evaluated.
- Can be used to evaluate the severity of allergic shock and differences between allergens subjected to physical transformations (ie, native versus processed).

### 2.2 | Limitations

- The occurrence of anaphylaxis is dependent on the mouse strain used: Balbc or C3H mice are prone to develop anaphylaxis, whereas C57BL/6 or A/J mice necessitate stringent exposure protocols to achieve sensitization.
- The clinical score may be biased as a consequence of the laboratory environment, stress level, animal strain, and technical experimenter.
- A decrease in temperature is only observed after a food/allergen challenge after a previous sensitization event; this endpoint therefore contains no predictive value for the sensitization potential of a food protein.

**TABLE 1** Table of studies measuring body temperature in allergic reactions

| Mouse/rat model    | Allergen          | Number of animals | $\delta T^{\circ}$ | Therapeutic or preventive strategy                                 | Conditions of measure                                                        | System of measurement                                           | Ref. |
|--------------------|-------------------|-------------------|--------------------|--------------------------------------------------------------------|------------------------------------------------------------------------------|-----------------------------------------------------------------|------|
| C3H/HeOuJ          | Whey              | N = 6-12          | 4°C                | Prevention with omega-3 long chain polyunsaturated fatty acids     | 1 h after challenge                                                          | Implantable electronic ID transponder                           | 11   |
| C3H/HeOuJ          | OVA               | N = 4-6           | 5°C                | Prevention with prebiotics: scGOS/IcFOS/pAOS                       | 30 min after challenge                                                       | Implantable electronic ID transponder                           | 12   |
| BALB/c             | Beta-lact         | N = 10            | 1.7°C              | Prevention with ratios of omega-6 and omega-3 fatty acids          | Before, 30 and 45 min after challenge                                        | Rectal probe                                                    | 13   |
| BALB/c             | Peanut, egg, milk | N = 3-5           | 3°C                | Anaphylaxis imaging                                                | Monitoring after challenge                                                   | Imaging method for whole-body surface temperature               | 14   |
| C3H/HeN            | Whey              | N = 12-15         | 4°C                | Microbiota composition and allergy protection                      | Before and 45 min after challenge                                            | Rectal probe                                                    | 15   |
| BN                 | OVA               | N = 8             | 3°C                | To develop an effective and rapid model of FA in Brown Norway rats | 60, 90 and 120 min after challenge                                           | Rectal probe                                                    | 16   |
| BALB/c             | BLG               | N = 5             | 5°C                | Nitration process of allergen                                      | Before and 15 and 30 min after iv                                            | Rectal probe                                                    | 17   |
| BALB/c             | OVA               | N = 10            | 0.5°C              | Microbiota composition and allergy protection                      | Before and 5 and 10 min after iv OVA challenge                               | Rectal probe                                                    | 18   |
| BALB/c             | OVA               | N = 5             | 1.5°C              | Anti-acid medication for risk of food allergy                      | Before and 15 min after challenge                                            | Rectal probe                                                    | 19   |
| BALB/c             | OVA               | N = 10            | None               | Heat process on allergen                                           | 30 min after challenge                                                       | Rectal probe                                                    | 20   |
| C3H/HeOuJ          | Whey              | N = 10            | 2-10°C             | Hydrolyze process on allergen                                      | Measurement of temperature over time: 0, 15, 30, 60, 120 min after challenge | A programmable temperature transponder implanted subcutaneously | 21   |
| C3H/HeOuJ          | Caseinate         | N = 6             | 5°C                | Transglutaminase cross-linked caseinate process                    | Measurement of temperature over time: 0, 15, 30, 60, after challenge         | A programmable temperature transponder implanted subcutaneously | 22   |
| BALB/cTac          | OVA               | N = 5-10          | 5°C                | Microbiota signature in allergy                                    | Measurement every 5 min: 5-60 min                                            | Rectal probe coupled to a Physitemp Thermalert Model TH-5       | 23   |
| BALB/c             | OVA               | N = 10            | 1°C                | To develop models of food allergy and oral tolerance               | 30 min after challenge                                                       | Rectal probe                                                    | 24   |
| C57BL/6            | Peanut            | N = 4-10          | 2°C                | Commensal bacteria for allergy protection                          | After challenge                                                              | Rectal probe                                                    | 25   |
| C3H/HeJ and BALB/c | Peanut            | N = 5             | 3-5°C              | Skin sensitization study                                           | 30 min after challenge                                                       | Rectal thermometer (WPI Instruments)                            | 26   |
| C3H/HeJ and BALB/c | OVA or peanut     | N = 5             | 4°C                | Epicutaneous immunotherapy                                         | 30 min after challenge                                                       | Rectal thermometer (WPI Instruments)                            | 27   |
| C3H/HeJ            | BLG               | N = 10            | 3°C                | Pasteurization process on milk allergen                            | After challenge                                                              | Rectal probe                                                    | 28   |

**TABLE 2** Studies using measurements of Igs from serum

| Mouse/rat model | Allergen                                                            | Sensitization and challenge                                                                    | Ig measured in serum                                                  | Aim of the study                                                                                                                                                                                                                                                                   | Ref. |
|-----------------|---------------------------------------------------------------------|------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------|
| BALB/c          | OVA                                                                 | I.g. + CT followed by i.g. challenge                                                           | IgG1, IgG2a: indirect ELISA<br>IgE: Ab-capture ELISA                  | To elucidate the class of bioactive polyphenols that exhibit a beneficial anti-allergic effect and to assess whether the protective effect matches the in vivo bioavailable metabolite concentrations                                                                              | 30   |
| BALB/c          | OVA,                                                                | I.p. followed by i.g.                                                                          | IgG1, IgG2a, IgA: indirect ELISA                                      | To investigate how thermal processing influences the ability of ovalbumin (OVA) to induce allergic symptoms and immune responses in a mouse model of food allergy                                                                                                                  | 20   |
| C57BL/6J        | OVA                                                                 | Oral (by feeding) + s.c. + alum followed by i.d. (ear test) challenge                          | IgG1: indirect ELISA<br>IgE: Ab-capture ELISA                         | To investigate the potential of hydrolyzed egg and whole egg to induce tolerance by the oral route (ie, by feeding)                                                                                                                                                                | 31   |
| BN              | OVA                                                                 | I.p. + alum + tBp followed by i.g. challenge                                                   | IgG1, IgG2a, IgG2b, IgA: indirect ELISA<br>IgE: Ab-capture ELISA      | To develop an effective and rapid model of food allergy with only one i.p. injection of the allergen with alum together with toxin from Bordetella pertussis (tBp) to promote IgE production and 2 wk later the i.g. administration of allergens                                   | 16   |
| BALB/c          | WPC, WPH, or BLG                                                    | I.p. with BLG + alum followed by oral (solution in drinking bottle) challenge with WPC and WPH | IgE: Ab-capture ELISA                                                 | To develop an experimental murine model of food allergy to the cow's protein $\beta$ -lactoglobulin (BLG) that mimics the main clinical characteristics of human disease as well as to examine the allergenic and immunological properties of extensively hydrolyzed whey proteins | 32   |
| BALB/c          | BLG                                                                 | I.p. + alum or FCA or FIA                                                                      | IgG1, IgG2a, IgE: indirect ELISA                                      | To study the sensitizing capacity of BLG and the influence of the use of adjuvant.                                                                                                                                                                                                 | 33   |
| BALB/c          | Whole milk                                                          | I.g. +/- CT followed by i.g. challenge                                                         | IgG1, IgG2a: indirect ELISA<br>IgE: EAST (enzyme allergosorbent test) | To map epitopes of the major soybean allergen Gly m 5 that are corecognized by casein-specific antibodies and to identify a peptide responsible for the cross-reactivity                                                                                                           | 34   |
| C3H HeOuJ       | WPC, ALA, BLG                                                       | I.g. + CT                                                                                      | IgG1, IgE: indirect ELISA                                             | To test a panel of high and low allergenic proteins                                                                                                                                                                                                                                | 35   |
| C3H HeOuJ PF    | WPC or WPH                                                          | I.g. + CT followed by i.g. + i.d. challenge                                                    | IgG1, IgE: indirect ELISA                                             | To validate a mouse model for cow's milk allergy to assess the potential allergenicity of hydrolyzed cow's milk-based infant formulas                                                                                                                                              | 21   |
| BN              | WPH, BLG                                                            | I.p. + alum                                                                                    | IgG2a, IgE: indirect ELISA<br>IgG1: inhibitory ELISA                  | To provide a thorough analysis of the immunogenicity and allergenicity of hydrolyzed cow's milk proteins for use in infant formulas                                                                                                                                                | 36   |
| BALB/c          | Extract                                                             | I.c. + CpG + CT or + non/CpG + CT followed by i.g. + CT challenge                              | IgG1, IgG2a, IgE, IgA: indirect ELISA                                 | To evaluate the effect of the application of peanut extract (PE) alone or mixed with CT and unmethylated sequences (CpG) as adjuvant on the intact skin                                                                                                                            | 37   |
| BALB/cJ         | Roasted extract or Ara h 1                                          | I.n. or e.c. followed by i.g. + CT challenge                                                   | IgG1: indirect ELISA<br>IgE: Ab-capture ELISA                         | To assess the impact of repeated short-term epicutaneous (e.c.) applications on intact skin or after repeated intranasal (i.n.) administration of food allergens from roasted peanut                                                                                               | 38   |
| BALB/c          | Ara h 1, Ara h 2, Ara h 3, Ara h 6, and Ara h 6 with no S-S bridges | I.p. + alum                                                                                    | IgG1, IgE: indirect ELISA (protein G for IgG removal)                 | To investigate the impact of heat processing of peanut seed on the sensitization to native Ara h 6                                                                                                                                                                                 | 39   |

(Continues)

TABLE 2 (Continued)

| Mouse/rat model | Allergen     | Sensitization and challenge          | Ig measured in serum                                                                                            | Aim of the study                                                                                                                                                                     | Ref. |
|-----------------|--------------|--------------------------------------|-----------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------|
| C3H/HeJ         | Extract      | I.g. + CT followed by i.p. challenge | IgG1, IgG2a: indirect ELISA<br>IgE, IgA: Ab-capture ELISA                                                       | To reveal the immune responses that are induced against peanuts allergens during sensitization, including the very early responses                                                   | 40   |
| C3H/HeJ         | Whole peanut | I.g. + CT followed by i.g. challenge | IgE: indirect ELISA                                                                                             | To develop a murine model of IgE-mediated peanut allergy that closely mimics human peanut allergy                                                                                    | 41   |
| BN              | Ara h 1      | I.p.                                 | IgG1, IgG2a: indirect ELISA and inhibitory ELISA for IgG1<br>IgE: Ab-capture ELISA<br>Total IgE: sandwich ELISA | To study the sensitizing capacity of four different 7S proteins and to determine whether related proteins would induce similar sensitization when removed from their "normal" matrix | 42   |
| BN              | Ara h 1      | I.p.                                 | IgG1, IgG2a: indirect ELISA<br>IgE: Ab-capture ELISA                                                            | To investigate the ability of digested protein—Ara h 1 to sensitize                                                                                                                  | 43   |

## 2.3 | Technical recommendations

- Using a rectal probe, mice or rats must be acclimated to the experimental room at least 1 hour before starting the temperature measurements to obtain stable values.
- The rectal temperature must be evaluated 10 minutes to 1.5 hours after the challenge.
- The animal temperature can be registered over time using a programmable temperature transponder implanted subcutaneously.

## 3 | EVALUATION OF IMMUNOGLOBULINS IN SERUM

While in vivo measurements are essential to assess the elicitation of an allergic response, they do not provide insight into de novo allergen sensitization. Therefore, blood, tissue, or organs must be collected and further analyzed by ex vivo methods. Serum immunoglobulin (Ig) content is the most common parameter measured when evaluating sensitization to food allergens in animal models, followed by fecal IgA (see Data S1), as antibody responses are considered a direct indicator of allergen sensitization together with mast cell and basophil degranulation. IgE is the most common Ig isotype measured when evaluating the allergenicity of food proteins and is regularly quantified in parallel with IgG1 (Table 2). Total and antigen-specific Ig levels can be analyzed, where the latter is a measure of how dosing with a given food or protein influences the overall level of IgE or IgG. Serum-specific IgE and IgG can be quantified by a series of different ex vivo methods, where ELISAs are the most commonly applied, followed by immunoblotting methods and mediator release assays (Figure 3). Whereas specific IgG in general is measured by means of an indirect ELISA,<sup>43</sup> specific IgE is most often measured by antibody-capture ELISA.<sup>44</sup> In fact, IgE is the least abundant Ig isotype in serum (with an approximate amount of only one IgE for every 50 000 IgGs<sup>45</sup>), making it difficult for IgE to compete for binding to proteins coated on ELISA plates. Other methods of measuring specific IgE include enzyme allergosorbent test (EAST) immunoblotting.<sup>34</sup> When measuring specific IgEs by means of in-house-developed antibody-capture ELISAs, there is a need for coupling the protein of interest to a molecule against which labeled secondary Igs are commercially available, as secondary Igs for direct binding to the proteins of interest can rarely be purchased. Molecules coupled to the protein of interest are most often digoxigenin (DIG)<sup>43</sup> or biotin,<sup>30</sup> with the additional advantage that they serve as signal amplifiers (Figure 3). Not only is the total level of specific Igs of interest in evaluating the sensitization response in animal models, the increase in affinity between Igs and the allergen is also important. Studies have shown that the binding strength between specific IgEs and the corresponding allergens is of great importance for the induction of a degranulation response and thereby the severity of the allergic

disease.<sup>46,47</sup> The avidity can be measured by means of simple potassium thiocyanate (KSCN) ELISAs which have shown that no general relationship exists between the level and avidity of specific Igs,<sup>48,49</sup> though a correlation may be observed during a multiple antigen exposure immune responses. This method, although not very sensitive, is based on the ratio of the areas derived from the curves obtained by plotting the OD and log of the sera dilution in the ELISA experiment with and without thiocyanate treatment. Where measures of specific IgE only allow for evaluation of sensitization, they provide no indication of the biological relevance of the IgEs present in the serum and thereby the clinical relevance of the food allergy model. To provide insights into the biological relevance of secreted IgEs, functional tests should be performed, such as the *in vivo* temperature drop, a skin prick test (SPT), or evaluation of challenge-derived symptoms. Further, *ex vivo* mediator release tests such as the rat basophilic leukemia (RBL) assay and basophil activation test (BAT) enable an evaluation of the biological relevance of the IgE raised in food allergy animal models (see Data S1 for description and opinion about mediator release assays and additional passive cutaneous anaphylaxis (PCA) and active cutaneous anaphylaxis (ACA) models).

### 3.1 | Strengths

- Specific IgE antibody analysis is the most trustworthy measure of sensitization.
- Measures of specific IgE antibodies are often used to evaluate not only sensitization but also the potential severity of the allergic reaction after a second encounter.
- Measurements of antibodies can be performed without the use of advanced equipment such as a cytometer or robotics.

### 3.2 | Limitations

- Assays often need to be developed in-house, restricting the possibilities for comparison between laboratories.
- IgE only accounts for a fraction of all serum antibodies, requiring more advanced ELISAs for analysis of specific IgE.
- IgE levels do not predict the clinical severity of a food allergy model, and other *ex vitro* experiments are needed to further address this parameter.

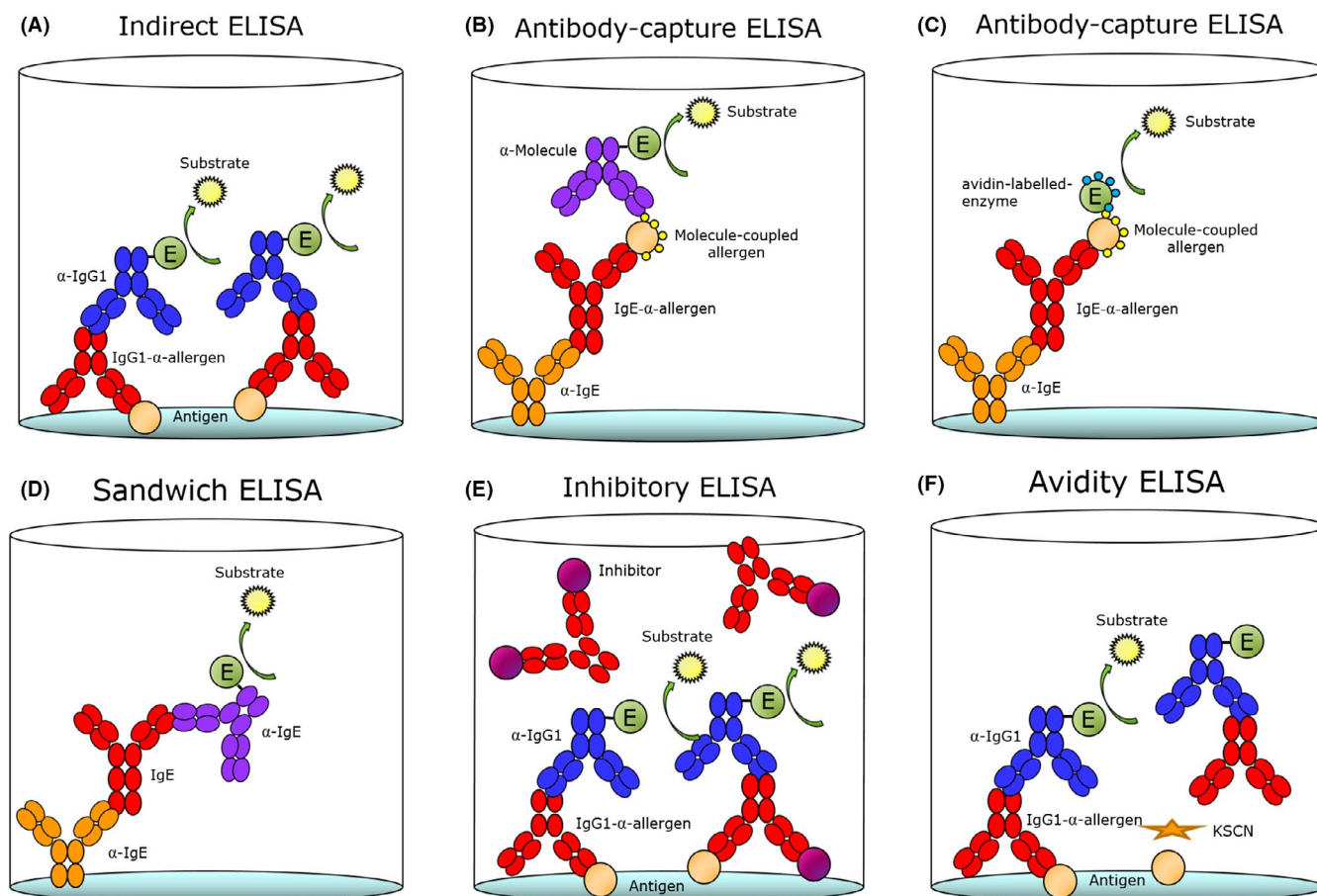


**FIGURE 3** ELISA methods. Antibodies (Abs) can be evaluated by means of different ELISA methods for assessment of their amount, specificity, and avidity. Specific IgG1 Abs are most often analyzed by means of an indirect ELISA (A), while specific IgE is most often analyzed by means of an Ab-capture ELISA (B, C). Total IgG1 and total IgE are analyzed by a sandwich ELISA (D). Furthermore, the specific Ab responses can also be evaluated for specificity with an inhibitory ELISA (E) or for binding strength with an avidity ELISA (F)

**TABLE 3** Studies using immune infiltrate as readout of allergic inflammation to egg, milk, and peanut proteins

| Mouse/rat Model   | Allergen                       | Allergen sensitization | Allergen challenge | Immune infiltrate                                   | Method                                                                                                                                                     | Therapeutic/preventive strategy                                                                                             | Ref. |
|-------------------|--------------------------------|------------------------|--------------------|-----------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------|------|
| C57BL/6 or BALB/c | OVA, crude peanut extract      | Skin                   | Intragastric       | Mast cells, eosinophils                             | Flow cytometry for intestinal eosinophils/mast cells, chloroacetate esterase staining for mast cells in jejunum, H&E staining                              | Anti-TSLP treatment or basophil depletion limits food allergen sensitization and the development of intestinal food allergy | 50   |
| C57BL/6           | OVA                            | Skin                   | Intragastric       | Mast cells, eosinophils                             | Flow cytometry for intestinal eosinophils/mast cells, chloroacetate esterase staining for mast cells in jejunum, H&E staining                              | Targeting basophil-derived IL-4 reduces food allergen sensitization and limits intestinal food allergy                      | 7    |
| BALB/c            | Raw or roasted peanut extracts | Skin                   | Intragastric       | Eosinophils                                         | Flow cytometry for eosinophils in the small intestinal lamina propria; H&E staining jejunum                                                                | NA                                                                                                                          | 51   |
| BALB/c            | OVA                            | Skin                   | Intragastric       | Eosinophils                                         | Flow cytometry for peripheral eosinophils; H&E staining jejunum                                                                                            | Basophil depletion attenuates intestinal allergy; CD4 T-cell depletion limits TSLP-mediated intestinal food allergy         | 52   |
| BALB/c            | OVA                            | Systemic               | Intragastric       | Mast cells, CD4 T cells                             | H&E staining jejunum, chloroacetate esterase staining of mast cells in the jejunum; flow cytometry of CD4 + T cells in the small intestinal lamina propria | Treatment with mast cell stabilizing cromolyn sodium protects against food allergen sensitization                           | 53   |
| BALB/c            | OVA                            | Skin                   | Intragastric       | Mast cells                                          | Chloroacetate esterase staining of connective tissue mast cells in the jejunum                                                                             | Targeting of IgE responses prevented intestinal mast cell expansion and anaphylaxis                                         | 54   |
| Various           | OVA                            | Intestine              | Intragastric       | Allergen-specific Tregs that acquire Th2 mast cells | Flow cytometry of small intestinal Foxp3 + Tregs; chloroacetate esterase staining of connective tissue mast cells in the jejunum                           | NA                                                                                                                          | 55   |
| BALB/c            | OVA; whole peanut extract      | Intestine              | Intragastric       | Mast cells; eosinophils                             | H&E staining jejunum; pinacyanol erythrosine staining to determine mast cell numbers and granulation status in the jejunum                                 | NA                                                                                                                          | 56   |
| BALB/c            | OVA                            | Skin                   | Intragastric       | Mast cells                                          | Chloroacetate esterase staining of mast cells in the jejunum                                                                                               | ST2 blockade attenuates food-induced anaphylaxis                                                                            | 57   |
| C3H/HeJ; BALB/c   | OVA; ground peanut             | Skin; systemic         | Intragastric       | Lap + Tregs                                         | Flow cytometry for Lap + Tregs in the lamina propria                                                                                                       | Clinical protection induced by epicutaneous immunotherapy (EPIT)                                                            | 58   |
| BALB/c            | BLG                            | Intestine              | Intragastric       | Lamina propria lymphocytes                          | ELISPOT IL-12, IL-17 producing lymphocytes from the intestinal lamina propria                                                                              | Blocking TSLP signaling prevents food allergy                                                                               | 59   |

- Measures with optical density (OD) as the unit only allow for one serum dilution.

### 3.3 | Technical recommendations

- Antibody-capture ELISAs should be used for the measurement of specific IgE.
- Other antibody parameters in addition to the amount of total and specific antibodies are relevant and should be measured, such as clonality and avidity.
- Measures of total and specific antibodies should always be expressed as titer values or as concentrations with no upper or lower limit for dilutions.
- Serum depleted of IgG using protein G columns before use in indirect ELISAs needs to be considered.

## 4 | PHENOTYPING OF T-CELL POPULATIONS

Assessment of serum Ig levels provides important information about the sensitization phase but does not allow for quantification of immune cell responses, including cellular infiltration to sites of allergic inflammation. The phenotyping of innate (eg, macrophages, eosinophils, basophils, neutrophils, dendritic cells) and adaptive (B and T cells) responses is indispensable for assessing the mechanisms of allergic sensitization (Table 3). Immune cells are generally isolated from organs, including the mesenteric lymph nodes, spleen, lung, skin, or intestine, and analyzed by flow cytometry. Typically, allergic inflammation is characterized by a predominantly type 2 immune response and secretion of the canonical type 2 cytokines IL-4, IL-5, IL-9, and IL-13 by innate immune cells (eg, eosinophils, basophils, mast cells (MCs), type 2 innate lymphoid cells, and polarized Th2 cells).<sup>44,45</sup> Indeed, in mice specifically expressing the ovalbumin T-cell receptor, sensitization to ovalbumin in their diet induced the expansion of IL-4-producing CD4<sup>+</sup> T cells in mesenteric lymph nodes, the spleen, and Peyer's patches.<sup>60</sup> Importantly, adoptive transfer of antigen-specific CD4<sup>+</sup> T cells derived from mesenteric lymph nodes of OVA-sensitized mice is sufficient to transfer allergen-induced diarrhea to naïve recipients. The recipient mice also display an up-regulation of the Th2-related chemokines CCL17 and CCL22 in the small intestine.<sup>61</sup> In addition to polarized Th2 responses, the proportion of other common T-cell subtypes, such as Th1 and Th17 that are characterized by the production of IFN- $\gamma$  and IL-17, respectively, can also be elevated in lymphoid organs of allergic mice. In contrast, expansion and/or the regulatory capacity of CD25<sup>+</sup> Foxp3<sup>+</sup> T cells associated with tolerance are often compromised in many food allergy models.<sup>62</sup> Additionally, other T-cell subtypes can be involved in food allergy pathogenesis. The recently discovered Th9 subset and associated IL-9 secretion were found to be involved in food allergy and especially in peanut allergies.<sup>63</sup> IL-9 is mainly responsible for the production of IL-4 by Th2 cells to promote mucosal mast cell

accumulation and secretion of mucus and chemokines by epithelial cells to sustain allergic inflammation.<sup>64</sup> To a lesser extent,  $\gamma\delta$ T cells found in the intestinal epithelium and in the lamina propria were also shown to be involved in food allergy. These cells are involved in blocking the induction of tolerance and modulating inflammatory responses.<sup>65,66</sup>

### 4.1 | Strengths

- Precise mechanistic insights into the cellular response in isolated organs and tissues support the sensitizing potential of food proteins when combined with additional readouts.
- Precise determination of the T-cell profile by using specific markers of the T-cell population.
- Quantitative evaluation of the infiltrating cell population by flow cytometry.

### 4.2 | Limitations

- Analysis of cell populations without the contribution of neighboring cell tissue (loss of microenvironment).
- Isolation of immune cells from tissues relies on enzymatic digestion protocols and may thus alter phenotypical and functional properties of the cells of interest.
- Difficulty with the separation of minor subpopulations.
- Sacrifice of the animal is required for organ and tissue sampling.
- Need for sophisticated equipment such as FACS.
- Type 2 immune response-associated mucus production in tissues makes cell isolation difficult and can create bias in cell phenotyping and frequencies.

### 4.3 | Technical recommendations

- Remove fat and store organs, tissues and cells at 4°C to avoid uncontrolled cell death or degradation of surface markers.
- Perform flow cytometry and culturing the same day as the animal kill.
- Phenotyping of T cells can be achieved by intracellular cytokine/transcription factor staining using flow cytometry.

## 5 | CYTOKINE PRODUCTION IN RESPONSE TO ALLERGEN RESTIMULATION

The logical follow-up to the analysis of infiltration/expansion of innate and adaptive immune cells in the tissues and organs is the evaluation of cytokine secretion. This evaluation comes directly from serum or from lymphatic tissue cells restimulated *ex vivo*. Food allergen stimulation of only lymphatic tissue cells, or in co-culture with dendritic cells, allows for the immunophenotyping of

the immune cell populations specific for the exposed food antigen or matrix. To confirm allergen specificity, splenocytes, mesenteric lymph node cells, or lamina propria cells isolated from sensitized and/or challenged mice are restimulated with corresponding allergenic proteins or peptides. After culture for up to 5 days, cytokines associated with the inflammatory response (IL-4, IL-5, IL-13, IL-17, and IFN- $\gamma$ ) and the regulatory response (IL-10 and TGF- $\beta$ ) are analyzed in the supernatants by ELISA<sup>60-64</sup> or a multiplex system. The cytokine production indicates whether T cells were primed toward the challenged food proteins and distinguishes Th1 or Th2 cell type responses. The prototypical type 2 cytokines include IL-4, IL-5, and IL-13. While IL-4 is critical for the polarization of Th2 cells and IgE class-switching in B cells,<sup>64</sup> IL-5 promotes the activation, proliferation, and survival of eosinophils, and IL-13 induces mucus production from goblet cells. Additional assays may be used including proteomics and gene expression profiling by PCR or microarray technology, that provide mechanistic insights and potential drug targets.

### 5.1 | Strengths

- Precise assessment of the allergen specificity by restimulating cells with the same allergen used in the animal model.
- Class determination of the T-cell response by evaluation of cytokine production in the supernatant of sorted T cells.
- Higher production of cytokines can be obtained after proliferation and restimulation with the antigen than by direct measurement in serum.

### 5.2 | Limitations

- Restimulation with allergens can activate nonspecific T cells due to certain cross-reactivity.
- Difficult to obtain a level above the sensitivity threshold with cells isolated from naïve mice.
- Some mechanistic endpoints are not equally important in animals and humans.

### 5.3 | Technical recommendations

- For allergen presentation, presorted T cells need to be co-cultured with dendritic cells.
- MHC peptide–tetramers can be used to sort specific T cells and have better assessment of allergen specificity.
- Need for positive (polyclonal anti-CD3/anti-CD28) and negative control (nonallergen) stimuli to ensure proper T-cell responsiveness.
- Endotoxin levels within the allergen extract need to be controlled to prevent bias in restimulation responses.

- Ideally, when using gene expression sequencing data, this method should be confirmed with at least one other technology (eg, flow cytometry).
- As cells and mediators associated with immune responses change rapidly, longitudinal assessments of mechanistic endpoints will be more informative than single time point assessments. The timing of the measurements will depend on the research question, for example, sensitization mechanisms vs mechanisms of acute allergic responses following (re)challenge.

## 6 | FUTURE ANALYSIS OF FOOD ALLERGY MODELS

To date, the methods to study intestinal pathophysiology are in vitro culture systems with cell lines or explanted mucosa grown in monolayers,<sup>67,68</sup> intestinal organoid cultures,<sup>69,70</sup> and “gut-on-a-chip” devices.<sup>71,72</sup> These technologies have offered many insights into gut physiology, but they lack cellular complexity, architecture, and immune and inflammatory responses that are crucial for a comprehensive understanding of underlying disease mechanisms and pathways. Alternatively, in vivo animal models provide the intact organ in the context of the vascular supply, systemic mediators, and circulating cells. However, in vivo experiments may be hampered by technical difficulties, including interindividual variability and maintenance of constant and reproducible experimental conditions.<sup>5</sup> To address the limitations of in vitro and in vivo models of gut disease, Yissachar et al<sup>73</sup> developed a chamber unit for culturing 12- to 14-day-old mouse colon or small intestine segments under highly controlled conditions. Of particular interest is that the chamber unit has two paired inputs and outputs that allow for controlled introduction of molecules or microbes into the lumen while simultaneously introducing continuous replenishment of medium to support tissue viability. The tissue remains intact, and the overall structure with epithelial cell layers is preserved for at least 24 hours, making this method suitable for studying epithelial transport of food allergens and their effect on epithelial integrity. However, other measurements are currently difficult due to the very short time that such tissue explants can be maintained. Furthermore, the enteric nervous system structure is maintained, and immune cells are detected as they found in healthy intestinal biopsies. It is possible to envisage the use of this type of ex vivo chamber unit in food allergy research by using intestinal fragments from naïve, sensitized, and allergic animals to introduce a variety of food proteins. It is thus possible to further elucidate pathways involved in luminal physiology and antigen uptake and presentation and make comparisons between known allergenic and nonallergenic proteins. This approach may lead to novel insights into new proteins and cross-reactive proteins and to the development of a predictive model for food allergy. Additional studies related to the survival and growth of anaerobic and aerobic microbiota revealed that the ex vivo colonization of cultured tissue with selected

microbes may be possible. Indeed, changes in the composition and metabolic activity of gut microbes can influence all aspects of innate and adaptive immune processes within the mucosa (see also Data S1 for stool consistency as a readout in food allergy assessment). Thus, focusing on the effect of diverse microbiota profiles and specific bacteria on immunological responses upon the introduction of allergenic proteins may lead to novel mechanisms, therapeutic targets, or predictive models. However, intra- and interlaboratory variability in microbiome composition and metabolic activity after birth as a result of the breeding environment is also a major underlying cause for conflicting results between experiments. This variability must be taken into account beforehand in the experimental design of an animal trial.<sup>5</sup> It is also noteworthy to consider the possible development of highly controlled chamber units for food allergy research used in combination with in vivo models to provide a new powerful strategy for studying mechanisms in the intestine.

## 6.1 | Strengths

- The tissue structure, cellular components, and neural system are highly preserved.
- The model provides the possibility to study immediate responses generated after the introduction of different molecules and microbes.

## 6.2 | Limitations

- Only short-term responses can be evaluated due to changes that can occur in the tissue over time.
- Currently, only intestinal segments from 12- to 14-day-old mice have been tested.
- Tissue preparation and assembly require specific skills.

## 7 | CONCLUSION

The recent broadening of our knowledge of food allergy pathogenesis and development of murine food allergy models has enabled us to model the allergic elicitation reaction as well as the preceding sensitization events and observe relevant symptoms with different food proteins (milk, egg, and peanut). The principal endpoint parameters described in this review are critical parameters that should be evaluated in a correct manner so that they may be powerful in the different rodent models.

Characterizing a food allergy model using temperature, level of IgS, phenotyping of the cell infiltrate, and cytokine production gives an overview of the reaction while providing us insight into the degree of sensitizing capacity of the allergen used. Nevertheless, even though the in vivo measurements and the ex vivo experiments provide us with many answers about the immune response and the

sensitization phase, we still do not have a complete overview of the immune mechanisms behind each reaction. There is still a strong need to better define the allergic reaction to predict the clinical outcomes of sensitization to novel food proteins. Although the current available models are suitable for studying the pathophysiology of food allergy, they still cannot predict the magnitude of the allergic potential of a particular allergen. Discovering and highlighting the molecules and cells involved in both sensitization and elicitation are necessary to improve risk assessment models and to facilitate the introduction of novel protein sources into our diet with a low risk of allergic sensitization.

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## CONFLICTS OF INTEREST

Dr Blanchard reports other from Nestec, outside the submitted work; Dr O'Mahony reports personal fees from AHL and grants from GSK, outside the submitted work. The other authors declare no conflict of interest.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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## Supplementary section

### Overview of *in vivo* and *ex vivo* endpoints in murine food allergy models: Suitable for evaluation of the sensitizing capacity of novel proteins?

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In this supplementary section, the *in vivo* and *ex vivo* endpoints not elaborated on or discussed in the main text are listed to complete the entire overview of murine model endpoints described in the literature for the allergenic foods milk, eggs and peanuts. Endpoints that are not often used and described in the literature, such as skin-specific responses, weight loss, ear swelling, flexiVent and plethysmography respiratory response measurements, and intestinal permeability responses have not been included in this supplementary section because no critical opinion could be given for these readouts.

#### Activity/behavior via camera

Behavioral changes as a result of a specific food allergen exposure in mice may be evaluated indirectly and typically includes recording of scratching or loss of mobility via camera. Such a noninvasive method to measure physical behavior without disturbing the animals allows for more objective analyses, without the human observer bias reported for the classical score system requiring subjective validation by the investigator (**Table S1**).

#### Strengths

- *Rapid assessment of allergic responses*
- *Provides more information than just sensitization potential as allergy effector mechanisms become activated*

- *Enables continuous data generation over a prolonged period of time, allowing longitudinal studies within one animal*

#### Limitations

- *Readouts are often subjective*
- *Not all observed responses are allergy-related and IgE-dependent*
- *The experimenter may easily bias manual counting of movements*

#### Technical recommendations

- *This endpoint requires an actual food/allergen challenge to observe sensitization events; this method has no predictive value for allergen sensitization*

### **Diarrhea and stool consistency**

Diarrhea is a classic clinical feature of food allergies, including peanut, egg and milk allergies. Oral allergen-induced diarrhea is mostly studied in OVA-challenged allergic mice (**Table S2**). In this model, systemic sensitization with OVA followed by an oral OVA challenge leads to OVA-specific diarrhea. Diarrhea is mostly assessed by visual monitoring of the mice up to 60 minutes after oral allergen administration and is defined as watery or liquid stool. Generally, there is no diarrhea observed during the sensitization phase, which only becomes apparent in the effector phase after the re-exposure to Ag that triggers the allergic response. The development of diarrhea requires the absorption of Ag, similar to that required to trigger an anaphylactic shock. Mast cells seem to play an important role in allergen-induced diarrhea, especially by synergistic signaling via serotonin and platelet-activating factor (PAF) (74). This mast cell activation by oral Ag challenge linked to diarrhea seems to be a local (intestinal) effect, as diarrhea can occur without systemic shock. Furthermore, there is increasing evidence that gut microbiota can influence allergenic diarrhea and that this can be potentially modulated by probiotics (75,76).

#### Strengths

- *Allows for an easy and rapid assessment of allergic elicitation reactions*

- *Apparent at low dosages of allergens (lower than needed to trigger shock)*

#### Limitations

- *Scoring is subjective to observer bias*
- *Assessment of watery stool is a readout of diarrhea that is not quantitative*

#### Technical recommendations

- *No predictive value for allergen sensitization as diarrhea only becomes apparent in the effector phase*

### **Anaphylaxis models (PCA/ACA)**

Two models to measure anaphylactic responses exist, namely, the cutaneous and systemic anaphylaxis models (**Table S3**). The latter was discussed in the main manuscript. In cutaneous anaphylaxis models, mice are sensitized by several injections with an allergen. In the passive cutaneous anaphylaxis (PCA) model, serum from these sensitized animals is injected into the dermis of naïve recipient mice, after which they are intravenously (i.v.) challenged with the allergen mixed with a dye. Because of the increased capillary permeability due to the local mast cell-IgE cross-linking and histamine release, extravasation of the dye is observed with the subsequent formation of a blue spot, which can be quantified. In the active cutaneous anaphylaxis (ACA) model, sensitized mice are i.d. challenged with allergen combined with an i.v. injection with dye to quantify localized allergic responses by using dye extravasation. Historically, a variety of experimental methods have been used, including injection of antigen prior to injection of the dye, injection of dye prior to injection of the antigen, and simultaneous injection of the dye and antigen. I.v. dye administration as a means for measuring allergic responses is a versatile assay because it can be used for measuring active, passive, and reverse passive local reactions. Numerous dyes have been utilized to assess allergic responses, including Trypan Blue, Pontamine Sky Blue, Evan's Blue, Geigy Blue 536, and India Ink. A solution of 0.5% Evan's Blue is currently the standard dye used for measuring allergic responses in the skin. Quantification of dye extravasation was originally obtained by measuring wheal size. Additionally, colorimetric analysis of

recovered dye from the ears or counts of degranulated mast cells can be quantified by excising skin tissue from the site of the reaction and staining with toluidine blue.

#### Strengths

- *Blue dye extravasation in ACA and PCA enables both visualization and relative quantification of a localized allergic reaction*
- *Animals are usually euthanized shortly after challenge, limiting any discomfort potentially caused by the allergen challenge (+/- dye administration)*

#### Limitations

- *I.v. and i.d. injections are technically challenging*
- *Evan's Blue dye is toxic*
- *The results are subjective to observer bias*
- *IgG1, IgG2a and IgG2b (but not IgG3) can induce PCA in mice, whereas in humans, there is no definitive evidence to date*
- *The anaphylaxis models are not suitable to predict sensitization because an actual food/allergen challenge is required*

#### Technical recommendations

- *Reliable results are dependent on consistent and successful ear and tail vein injections; if the entire challenge dose is not administered into the ear, if ear injection results in obvious puncture of a blood vessel, or if the Evan's Blue i.v. injection is not complete, the animal should be excluded from analysis*
- *PBS injection in ACA/PCA should routinely be used as an internal control*
- *Injection to the back skin of mice may be less technically challenging, and, in addition, it allows for the testing of multiple antigens simultaneously*

### **Basophil/mast cell activation test (BAT)**

Mast cells (MCs) are innate immune cells derived from CD34<sup>+</sup> progenitor stem cells in bone marrow (77,78). MCs enter circulation as immature cells and undergo maturation within

peripheral tissues, such as the skin and gastrointestinal tract. Maturation and homing are strongly affected by the local tissue microenvironment, the presence of stem cell factor and lipoteichoic acid and critical MC transcription factors that regulate phenotype and cell fate (79,80). Antigen-specific IgE cross-linking on MCs leads to the release of proteases and various inflammation mediators, such as histamine, prostaglandins and serotonin. Serum mast cell protease MCPT-1 is commonly used as a systemic readout for MC activation in mouse food allergy models, although its levels may vary depending on the genetic background. Factors influencing MC tissue homing and methods used for MC analysis are described in **Table S4**. Basophils are granulocytes developed from CD34+ multipotent hematopoietic stem cells that, contrary to MCs, undergo maturation in the bone marrow (81). Basophils, similar to MCs, rapidly secrete allergic inflammatory mediators upon allergen-IgE cross-linking of Fc fragments on their cell surface. The basophil activation assay (BAT), currently used primarily in humans instead of murine allergy models, has been suggested as a new diagnostic test for food allergy with the aim of replacing the oral food challenge, currently the gold standard for food allergy diagnosis in humans (82). The BAT is an *in vitro* flow cytometry-based functional test that determines the degree of basophil activation after exposure to specific allergen stimuli by changes in cell surface membrane markers such as CD63, IgE/CD203c, CCR3/CD3, CD123/HLA-DR (83), which can be rapidly assessed to measure human basophil activation. In contrast to IgE-mediated anaphylaxis in humans, both IgG and IgE can play a role in murine anaphylaxis. Murine specific basophil activation markers include CD200R1 (84) and CD41(85) for IgE-mediated activation, while CD200R3 can be used as an IgG1-specific activation marker (86). Its sensitivity and specificity differs for different allergens, and the standardization of results between laboratories is still challenging (82,84,87).

### Strengths

- *MCPT-1 is generally used as a systemic readout of MC activation in mouse food allergy models*

- *The BAT has good diagnostic accuracy in food allergy diagnosis without the need to perform stressful and sometimes fatal oral food challenges*
- *Detection of  $\beta$ -hexosaminidase in MCs can be performed colorimetrically, which is more favorable in terms of costs compared to those of an ELISA*

#### Limitations

- *Mature MCs are limited to tissues and hardly circulate in peripheral blood, which makes it difficult to obtain sufficient quantities for in vitro tests*
- *MCPT-1 induction may vary with the mouse genetic background; combination with other readouts is envisaged to describe anaphylactic status*
- *BAT standardization between laboratories is challenging*

#### Technical recommendations

- *The BAT assay needs to be performed within 4 hours of drawing blood*

### **Mucus/fecal IgA**

Early studies proposed fecal IgA as a noninvasive biomarker of food tolerance. Often measured as total IgA, the specificity of the effect toward allergen uptake is debatable. A decrease in specific secretory IgA has been observed in most food allergy models (**Table S5**), but specific secretory IgA is not required for oral tolerance acquisition after food exposure in the absence of sensitization (88). Interestingly, this finding may be because the development of allergen-specific anti-OVA IgA in intestinal lavage seems to require oral exposure and (oral) sensitization since intraperitoneal sensitization alone does not seem sufficient to mount such an intestinal-specific IgA response (33,44). While the level of intestinal lavage IgA varies in function of the allergen used for sensitization, IgG1 and IgE alone or combined seemed to have more discriminating potency for different allergens or processing of allergens (milk proteins). Notably, the presence of IgA in feces is highly linked to the gut microbial composition (44). In a peanut allergy model, mucosal IgA responses induced by commensal bacteria were essential in preventing intestinal inflammation; fecal

IgA might thus be a potential contributor of gut microbiota-mediated immune exclusion, possibly reducing systemic allergen uptake (51).

#### Strengths

- *IgG1 and IgE alone or combined seem to have more discriminating potency for different allergens or processing of allergens*
- *Fecal IgA as a potential contributor to gut microbiota-mediated immune exclusion*

#### Limitations

- *The presence of IgA in feces is highly linked to the gut microbial composition*

#### Technical recommendations

- *Fecal/intestinal total or specific IgA could be used as additional markers of interest for allergen sensitization capability but are not sufficient today as standalone markers*

**Table S1:** Literature covering activity/behavior via camera.

| Mouse/rat model        | Allergen                 | Number of animals | Therapeutic or preventive strategy                                                                         | System of measurement                                                           | Conditions of measure                                                | Ref  |
|------------------------|--------------------------|-------------------|------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------|----------------------------------------------------------------------|------|
| C3H/HeO <sub>u</sub> J | Whey                     | N=20              | Effects of an immune response on behavior and neuronal activation                                          | Video tracking software                                                         | 24 and 48 h after last sensitization and 24 h after challenge        | (89) |
| BN rats                | OVA                      | N=24              | Development of an effective food allergy model                                                             | Activity meter recording movements for a 21-min period                          | 24 h before induction and immediately after challenge                | (44) |
| C3H/HeN                | BLG                      | N=30              | Impact of gut microbiota on allergic manifestations                                                        | Scratching and mobility loss scoring by protocol-blinded investigators          | 15-45 min. after challenge                                           | (43) |
| BALB/c                 | Cow's milk               | N=18              | Cross-reactivity <i>in vivo</i> study using a food allergy model                                           | Responses assessed by two independent investigators                             | 30 min. after challenge                                              | (90) |
| BALB/c                 | OVA                      | N=20              | Development of food allergy and oral tolerance models                                                      | Reactions monitoring by two independent individuals                             | between 30 to 60 min. postchallenge                                  | (88) |
| BALB/c                 | OVA, peanut              | N=6-8             | Testing the immunologic mechanisms of intestinal food allergy predisposition by epicutaneous sensitization | Clinical symptoms scoring investigated by treatment-blinded investigators (0-6) | Individual observations for 30 min. starting 20 min. after challenge | (64) |
| BALB/c                 | Peanut, cow milk, potato | N=32              | Food protein extracts allergenicity evaluation                                                             | Monitoring using symptoms pre-established scale                                 | 10 min prior and in 10 min. intervals (up to 50) after the challenge | (91) |
| BALB/c                 | Casein, peanut, OVA      | N=32              | Automated noninvasive physical activity imaging                                                            | 20 min. of video camera imaging                                                 | Immediately after challenge                                          | (16) |
| C3H/HeJ                | Peanut                   | N=5               | Immunotherapy with allergen nanoparticles                                                                  | Visually observed symptoms scoring (0-5)                                        | 30 to 40 min. after challenge                                        | (92) |
| BALB/c                 | Lactoferrin              | N=60              | Dose-dependent allergenic properties measurement                                                           | Visually observed symptoms scoring (0-5)                                        | 30 min. after injection                                              | (93) |

**Table S2:** Studies using diarrhea as a readout for allergic reactions.

| Mouse/rat model | Allergen | Number of animals | Conditions of measure | System of measurement                                                                                                         | Ref  |
|-----------------|----------|-------------------|-----------------------|-------------------------------------------------------------------------------------------------------------------------------|------|
| BALB/c          | OVA      | N=5-6             | 1 h after challenge   | Diarrhea was assessed by visually monitoring mice. Mice with profuse liquid stool were recorded as diarrhea-positive animals. | (94) |
| BALB/c          | OVA      | N=15-16           | 1 h after challenge   | Diarrhea was assessed by visually monitoring mice. Diarrhea was defined as the development of watery green stools.            | (74) |
| BALB/c          | OVA      | N=5               | 1 h after challenge   | Diarrhea was assessed by visually monitoring mice. Diarrhea was defined as the development of watery green stools.            | (95) |
| BALB/c          | OVA      | N=12              | 1 h after challenge   | Diarrhea was assessed by visually monitoring mice. Mice with profuse liquid stool were recorded as diarrhea-positive animals. | (75) |
| BALB/c          | OVA      | N=27-34           | Not specified         | Not specified                                                                                                                 | (76) |

**Table S3:** Studies implementing anaphylactic models as a readout for allergy sensitization.

| Mouse/rat model | Allergen               | Number of animals | Sensitization                                                      | Challenge                                                                | Conditions of measure      | System of measurement | Ref   |
|-----------------|------------------------|-------------------|--------------------------------------------------------------------|--------------------------------------------------------------------------|----------------------------|-----------------------|-------|
| BALB/c          | Peanut agglutinin; OVA | N=5               | I.d. pooled allergic serum samples in ears of naïve recipient mice | After 48 hours: i.v. allergen + Evan's Blue dye                          | 30 minutes after challenge | Dye extravasation     | (96)  |
| BALB/c          | Peanut agglutinin; OVA | N=5               | I.d. pooled allergic serum samples in ears of naïve recipient mice | After 48 hours: i.v. allergen + Evan's Blue dye                          | 30 minutes after challenge | Dye extravasation     | (97)  |
| BALB/c          | BLG, Ara h1, Ara h2    | N=5               | I.d. allergic serum samples in ears of naïve recipient mice        | After 48 hours: i.v. allergen + Evan's Blue dye                          | 30 minutes after challenge | Dye extravasation     | (98)  |
| BALB/c          | OVA                    | N=15              | I.p. OVA with alum                                                 | S.c. injection with OVA, followed by i.v. injection with Evan's Blue dye | 30 minutes after challenge | Dye extravasation     | (99)  |
| ICR             | BSA                    | N=5               | S.c. daily injection with D-galactose for 6 weeks                  | I.d. injection with BSA in shaved back & i.v. Evan's Blue dye            | 20 minutes after challenge | Dye extravasation     | (100) |

|          |         |        |                                                      |                                                                       |                                 |                                  |       |
|----------|---------|--------|------------------------------------------------------|-----------------------------------------------------------------------|---------------------------------|----------------------------------|-------|
| BALB/c   | OVA     | N=5-6  | 7x i.p. OVA with alum                                | I.v. injection Evan's Blue, followed by i.d. injection of OVA in ears | 30 minutes after challenge      | Dye extravasation                | (101) |
| BALB/c   | OVA     | N=9-10 | I.p. OVA with alum (IgE) or i.p. OVA with CFA (IgG1) | I.d. ear injection of OVA + i.v. injection of Evan's Blue             | 30 minutes after challenge      | Dye extravasation                | (102) |
| 129/Sv   | TNP-OVA | N=5-7  | I.v. IgE anti-TNP                                    | After 24 hours: i.v. injection of TNP <sub>4</sub> -OVA               | 0 – 60 minutes after challenge  | Clinical symptoms of anaphylaxis | (103) |
| C57BL/6J | OVA     | N=7-15 | 6x OVA i.p.                                          | I.p. injection of OVA                                                 | 0 – 120 minutes after challenge | Clinical symptoms of anaphylaxis | (104) |
| ICR      | BSA     | N=5    | S.c. daily injection with D-galactose for 6 weeks    | I.v. injection with BSA                                               | 0 – 60 minutes after challenge  | Clinical symptoms of anaphylaxis | (100) |

**Table S4:** Studies and methods used for analyzing mast cell (MC) activation and recruitment.

| Mouse/rat model                                          | Allergen | Number of animals | Food allergy induction and/or mast cell (MC) detection                                                                                                                        | MC maturation and activation measurement                                                                                                     | Key messages                                                                                                                                            | Ref   |
|----------------------------------------------------------|----------|-------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------|-------|
| Germ-free and conventional C57BL/6J, Kit <sup>W-sh</sup> | -        | Not specified     | Histological staining: toluidine blue, flow cytometry of skin tissue cells stained with anti-CD117, -FcεRI.                                                                   | <i>In vivo</i> MC activation of GF and CV mice using degranulation-provoking compound 48/80. qPCR on laser-dissected MCs                     | GF mice have immature MCs in the dermis; skin microbiome drives SCF production in keratinocytes, which triggers the homing and maturation of dermal MCs | (105) |
| BALB/c                                                   | OVA      | N=6               | Epicutaneous sensitization with OVA followed by i.g. doses of OVA<br>Immunohistochemical: MCPT-1+ cells                                                                       | Mast Cell Protease-1 (MCPT-1) in sera by ELISA                                                                                               | Adjuvant-free model of OVA food allergy was established                                                                                                 | (106) |
| BALB/c                                                   | OVA      | N=10              | I.p. sensitization with OVA+alum followed by i.g. doses of OVA                                                                                                                | MCPT-1 in sera by ELISA                                                                                                                      | Thermal processing of OVA decreased its allergenicity                                                                                                   | (107) |
| BN rats                                                  | OVA      | N=20              | I.g. doses of OVA for 6 weeks (adjuvant-free)<br>Histological staining: toluidine blue, electron microscopy                                                                   | qPCR, Western blot, Ca <sup>2+</sup> imaging, immunofluorescence of store-operated calcium channels on MCs obtained by peritoneal lavage     | OVA-induced food allergy upregulated Ca <sup>2+</sup> entry through SOCs, thereby leading to subsequent mast cell activation and degranulation          | (48)  |
| Germ-free, conventional and gnotobiotic BALB/c           | OVA      | N=11-14           | I.p. sensitization with OVA+alum followed by i.g. doses of OVA<br>Histological staining: pararosaniline, Flow cytometry of skin tissue cells stained with anti-CD117, -FcεRI. | <i>In vivo</i> MC activation of GF and CV mice using degranulation-provoking compound 48/80. MCPT-1 in sera and jejunum homogenates by ELISA | Germ-free mice don't develop symptoms of food allergy. Intestinal microbiota is necessary for MC maturation and homing to the gut.                      | (108) |

**Table S5:** Studies using mucus/fecal IgA measures as a readout for allergy sensitization

| Mouse/rat model | Allergen | Number of animals | Exposure route                                                                            | Conditions of measure                                                                                                                                                            | System of measurement       | Observed effects                         | Ref   |
|-----------------|----------|-------------------|-------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------|------------------------------------------|-------|
| BN              | OVA      | N=8               | Intraperitoneal injection vs injection plus oral gavage daily for 3 weeks (5 weeks total) | Serum sIgA at day 0, 14, 21, 28, 35 and 42; gut washes from sacrificed rats 2 days after anaphylactic response; IgA mRNA from small intestine 2 days after anaphylactic response | Indirect ELISA & Taqman-PCR | sIgA levels decreased                    | (44)  |
| BN              | OVA      | N=10              | Oral gavage daily for 6 weeks                                                             | Gut washes and small intestinal tissue biopsies from sacrificed rats 1.5 h after challenge                                                                                       | ELISA                       | No effects; sIgA increased by probiotics | (109) |
| C3H/HeJ         | ALA BLG, | Not indicated     | Oral gavage on                                                                            | Intestinal lavage IgA                                                                                                                                                            | ELISA                       | sIgA levels were                         | (33)  |

|                                                 |        |       |                                                                              |                                                                                                                                                              |       |                         |      |
|-------------------------------------------------|--------|-------|------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------|-------|-------------------------|------|
|                                                 | casein |       | days 0, 7, 14, 21, 28 and 35                                                 | content was measured after anaphylactic response challenge on day 42                                                                                         |       | decreased               |      |
| C57BL/6<br>,<br>C57BL/6<br>Foxp3 <sup>gfp</sup> | Peanut | N=4-8 | Intragastric sensitization in combination with antibiotics and/or Clostridia | Fecal IgA in peanut-sensitized mice (pathogen-free, <i>Clostridium</i> , <i>B. uniformis</i> or conventional treated mice) was measured 24 h after challenge | ELISA | IgA levels were reduced | (51) |

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